

REMARKS/ARGUMENTS

Status of the claims

Claims 1-19 and 30-46 are pending and under examination. Claims 24 and 26-29 are canceled without prejudice to subsequent revival.

Claims 1-3 and 7-8 have been amended in the manner suggested by the Examiner. These amendments add no new matter and are for the purpose of providing improved clarity. Support for these amendments can be found in the claims as filed.

Claims 30-46 have been added. Claims 30-43 are directed to methods of producing and methods of transforming transgenic alkaloid producing plants of the *Papaver Somniferum* species comprising the step of adding a buffering agent which maintains the pH of the plant material or culture medium for culturing the plant material within a range of pH 5.5 to pH 6.5 during transformation and/or culturing of the plant cells. Claims 44-46 are directed to methods of producing and methods of transforming transgenic alkaloid producing plants comprising the step of adding a buffering agent which prevents, reduces the rate of, or delays a rise in pH of the plant material or culture medium from a desired pH level of between pH 5.5 and 6.5 during transformation and/or culturing of the plant cells. These claims add no new matter. Support can be found, for example, in the claims as filed and in the specification on page 7, lines 1-8.

Drawings

Applicants filed corrected drawings June 9, 2003.

The Present Invention

The present invention provides, *inter alia*, methods of producing transgenic alkaloid producing poppy plants. Before the advent of the present invention, it was known to be difficult to produce viable transgenic poppies having high alkaloid content. The present inventors discovered that there is an unexpected and rapid rise in the pH of the culture medium when poppy cells are cultured and that this rise is at least partly responsible for poor transformation efficiency. By including a pH buffering agent that prevents, reduces or delays

a rise in the pH of the plant material and/or culture medium during transformation or culturing of the plant material, the inventors are able to achieve higher transformation efficiency. The inventors thus solved a problem associated with the production of transgenic poppy plants.

Rejection under 35 U.S.C. §112, first paragraph

Claims 1-5, 7-19, 24, 26, 28 and 29 remain rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification, while being enabling for a method of producing a transgenic *Papaver somniferum*, does not reasonably provide enablement for a method of producing any transgenic poppy plant.

Applicants respectfully traverse this rejection because there is no evidence of record suggesting that a skilled practitioner would be unable to perform the claimed methods.

The initial burden to support an enablement rejection rests on the Examiner. In this regard, § 2164.04 of the MPEP states: “the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993)(examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure).” MPEP § 2164.04 further states that a specification must be taken as being in compliance with the enablement requirement of 35 U.S.C. § 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).” The MPEP quotes *Marzocchi* as follows: “it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure. 439 F.2d at 224, 169 USPQ at 370.”

The present rejection does not meet the *Marzocchi* standard, as articulated in the MPEP. Rather, the Action sets forth only a general assertion that there the claimed method will not work for producing any transgenic alkaloid producing poppy plant and cites Hansen

et al. (*Trends in Plant Science*, 1999, 4(6):226-230) for the proposition that plant transformation is an art. A closer reading of the article reveals, however, that plant transformation technology has become commonplace and that the challenges in plant transformation are related to improving the existing transformation techniques to provide efficient and predictable transgene expression (see abstract). Notably, neither the Action nor the cited reference provides any evidence that a skilled practitioner would be unable to transform a variety of poppy plant species using conventional methods of plant transformation and regeneration with the addition of the novel steps of the present invention, *i.e.*, the addition of a buffering agent to prevent, reduce, or delay the rise in pH of the plant material or culture medium. In fact, a review of the available literature demonstrates otherwise. For example, Applicants submit as Exhibit A an article by Park *et al* from the peer reviewed journal of *Plant Physiology* (2002, 128, 696-706) describing the transformation of California Poppy, a poppy plant of the *Eschscholtzia* species. The article provides evidence that the skilled practitioner is capable of using conventional techniques to transform poppy plants. As stated in the specification, methods of the invention are based on the use of conventional methods of plant transformation and regeneration techniques but include the additional step of stabilizing the pH of the medium. The Action provides no reason to believe that a skilled practitioner would be unable to use conventional methods such as those described in the Park *et al.* article with the methods of the present invention to transform an alkaloid producing poppy plant. Accordingly, absent any evidence to the contrary, the Action provides no reason to believe that a skilled practitioner, armed with the knowledge in the art and the teachings of the present specification, would be unable to perform the claimed methods. Absent such evidence, Applicants respectfully submit that the rejection for alleged lack of enablement is improper and request that it be withdrawn

Rejections under 35 U.S.C. § 102(b)

The rejection of claims 1-9, 11-13, 16, 17, 24, and 26-29 under 35 U.S.C. § 102 is improper. Although the Action states that the claims of the present invention are anticipated by Yoshimatsu *et al.* (*Biotechnology in Agriculture and Forestry* 1996, 38, 243-252) the Action fails to identify any disclosure or suggestion of the method steps of the present invention.

DOCKET NO.: J&J 1764
Application No.: 09/600,025
Office Action Dated: March 5, 2003

PATENT
REPLY FILED UNDER EXPEDITED
PROCEDURE PURSUANT TO
37 CFR § 1.116

For a rejection under § 102(b) to be properly founded, a single prior art reference must disclose, either expressly or inherently, each and every element of the claimed invention. *See, e.g., Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Verdegaal Bros. V. Union Oil Co. Of California*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). In *Scripps Clinic & Research Found. v. Genentech, Inc.*, 18 USPQ2d 1001 (Fed. Cir. 1991), the Federal Circuit held that there can be no difference between an anticipatory reference and the claimed subject matter:

Invalidity for anticipation requires that all of the elements and limitations of the claim are found with a single prior art reference. . . . There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. *Id.* at 1010.

Anticipation can be found, therefore, only when a cited reference discloses all of the elements, features, or limitations of the claimed invention.

Claims 1-9, 11-13, 16, 17, 24, and 26-29 require that the transformation of alkaloid producing poppy plant material and/or the culturing of the transformed plants material occur in the presence of a buffering agent that prevents, reduces the rate of, or delays the rise in pH of the plant material or culture medium from a desired pH level. It is the Action's position that since the Yoshimatsu reference contemplates the use of MS medium or Woody Plant medium in its transformation protocol and/or culturing protocol, its methods anticipate the present invention because of the inherent properties of MS or Wood Plant medium.

Applicants submit, however, that the Action does not establish inherency as required by MPEP § 2112 and the courts.

As stated in MPEP § 2112 and the Court of Appeals for the Federal Circuit in *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (1993),

The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic.

Instead, as also stated in MPEP § 2112 and the Court of Appeals for the Federal Circuit in *In re Robertson* 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (1999),

To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill.'

The claims at issue require not only the presence of a buffering agent but the ability of the buffering agent to prevent, reduce the rate of, or delay the rise in pH of the plant material or culture medium. Accordingly, it is not enough that a buffering agent be present, the buffering agent **must** be able to prevent, reduce the rate of, or delay the rise in pH of the plant material or culture medium. The Yoshimatsu reference does not explicitly require that the MS or Woody Plant media be added in an amount sufficient to prevent, reduce the rate of, or delay the rise in pH of the plant material or culture medium, nor does it inherently disclose that the MS media or Woody Plant media is present in an amount sufficient to prevent, reduce the rate of, or delay the rise in pH of the plant material or culture medium. As explained in MPEP § 2112 and described by the court, “in relying upon the theory of inherency, the Examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the prior art.” *Ex Parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Int. 1990). In the present rejection, although it has been stated that MS media comprises ammonium nitrate and potassium nitrate, this in no way provides any basis that the media is sufficient to prevent, reduce the rate of, or delay the rise in pH of the plant material or culture medium.

The inventors of the present application surprisingly discovered the non-obvious source of a problem - pH rise in alkaloid producing plant culture medium during transformation - and solved the problem by adding a buffer that is capable of preventing, reducing, or delaying the rise in pH. The cited art fails both to recognize the problem or to suggest the source of the problem and provides no teachings that buffers be added during transformation in an amount sufficient to adjust pH level to the desired degree. Clearly, before the advent of the present invention, one of ordinary skill in the art would not have recognized that desirability of preventing, reducing, or delaying the rise in pH because it was not even known that such a rise in pH occurs. Thus, Applicants submit that anticipation has not been established. Absent evidence to the contrary, the rejection under §102(b) is improper and should be withdrawn.

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37 CFR § 1.116

Rejections under 35 U.S.C. § 103

Claims 14 and 15 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Yoshimatsu *et al.*, in view of Bidney (U.S. Patent No. 5,932,782). As previously discussed, the present inventors discovered the non-obvious source of a problem of poor transformation efficiencies of alkaloid plants and solved the problem by teaching a step of preventing, reducing, or delaying a rise in pH of the plant material or culture medium for transformation or culturing of the plant material. The Yoshimatsu reference fails to teach or suggest the source of the problem, *i.e.*, the rise in pH, or the solution to the problem, *i.e.*, the addition of buffering agent to prevent, reduce or delay the rise in pH. The Bidney reference fails to supply the missing teaching as the Bidney reference also neither teaches nor suggests transforming alkaloid producing poppy plant material and/or regenerating a transgenic alkaloid producing poppy plant from such material in the presence of a buffering agent to prevent, reduce the rate of or a delay a rise in pH. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. §103(a) be withdrawn.

Applicants respectfully submit that the present application is in condition for allowance. Favorable consideration and an early notice of allowance are respectfully requested.

Date:



1/15/04

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Antisense RNA-Mediated Suppression of Benzophenanthridine Alkaloid Biosynthesis in Transgenic Cell Cultures of California Poppy¹

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California poppy (*Eschscholzia californica* Cham.) cell cultures produce several benzophenanthridine alkaloids, such as sanguinarine, chelirubine, and macarpine, with potent pharmacological activity. Antisense constructs of genes encoding two enzymes involved in benzophenanthridine alkaloid biosynthesis, the berberine bridge enzyme (BBE) and *N*-methylcoclaurine 3'-hydroxylase (CYP80B1), were introduced separately into California poppy cell cultures. Transformed cell lines expressing antisense BBE or antisense CYP80B1 constructs and displaying low levels of BBE or CYP80B1 mRNAs, respectively, showed reduced accumulation of benzophenanthridine alkaloids compared with control cultures transformed with a β -glucuronidase gene. Pathway intermediates were not detected in any of the transformed cell lines. The suppression of benzophenanthridine alkaloid biosynthesis using BBE or CYP80B1 antisense RNA constructs also reduced the growth rate of the cultures. Two-dimensional ¹H-nuclear magnetic resonance and *in vivo* ¹⁵N-nuclear magnetic resonance spectroscopy showed no difference in the abundance of carbohydrate metabolites in the various transgenic cell lines. However, transformed cells with reduced benzophenanthridine alkaloid levels contained larger cellular pools of several amino acids including alanine, leucine, phenylalanine, threonine, and valine compared with controls. The relative abundance of tyrosine, from which benzophenanthridine alkaloids are derived, was less than 2-fold higher in antisense-suppressed cells relative to controls. These results show that alterations in the metabolic flux through benzophenanthridine alkaloid biosynthesis can affect the regulation of amino acid pools. These data provide new insight into the metabolic engineering of benzophenanthridine alkaloid pathways.

Alkaloids are a diverse group of low- M_r , nitrogenous compounds found in about 20% of plant species. Many of the approximately 12,000 alkaloids for which structures have been described function in the defense of plants against herbivores and pathogens. The potent biological activity of some alkaloids has also led to their widespread use as pharmaceuticals. The benzylisoquinoline alkaloid class, in particular, includes several important medicinal compounds such as morphine, codeine, papaverine, berberine, (+)-tubocurarine, and sanguinarine. All benzylisoquinoline alkaloids share a common biosynthetic origin beginning with a lattice of decarboxylations, *ortho*-hydroxylations, and deaminations that convert L-tyrosine into both dopamine and 4-hydroxyphenylacetaldehyde. Dopamine and 4-hydroxyphenylacetaldehyde condense to form the trihydroxylated

alkaloid (S)-norcoclaurine (Fig. 1), which is the central precursor to all benzylisoquinoline alkaloids produced in plants (Stadler et al., 1987, 1989). (S)-Norcoclaurine is converted to (S)-reticuline by a 6-O-methyltransferase (Morishige et al., 2000), an N-methyltransferase (Choi et al., 2001), a P450 hydroxylase (Pauli and Kutchan, 1998), and a 4'-O-methyltransferase (Morishige et al., 2000). The aromatic-ring hydroxylation involved in the conversion of (S)-norcoclaurine to (S)-reticuline originally was thought to be catalyzed by a nonspecific phenol oxidase (Loeffler and Zenk, 1990). However, a P450-dependent monooxygenase (CYP80B1) isolated from California poppy (Pauli and Kutchan, 1998) exhibits a K_m for (S)-N-methylcoclaurine considerably lower than that of the phenolase; thus, CYP80B1 is now known to convert (S)-N-methylcoclaurine to (S)-3'-hydroxy-N-methylcoclaurine (Fig. 1).

(S)-Reticuline is a branch-point intermediate involved in the biosynthesis of many structural types of benzylisoquinoline alkaloids. One such group, the benzophenanthridine alkaloids, includes the antibiotics sanguinarine, which is used in various oral hygiene products, and macarpine, the most highly oxidized alkaloid in its class. The first committed step in benzophenanthridine alkaloid biosynthesis involves conversion of the *N*-methyl group of (S)-reticuline into the methylene bridge moiety of (S)-scoulerine by the BBE (Fig. 1). (S)-Scoulerine is then converted by two P450-dependent oxidases, an

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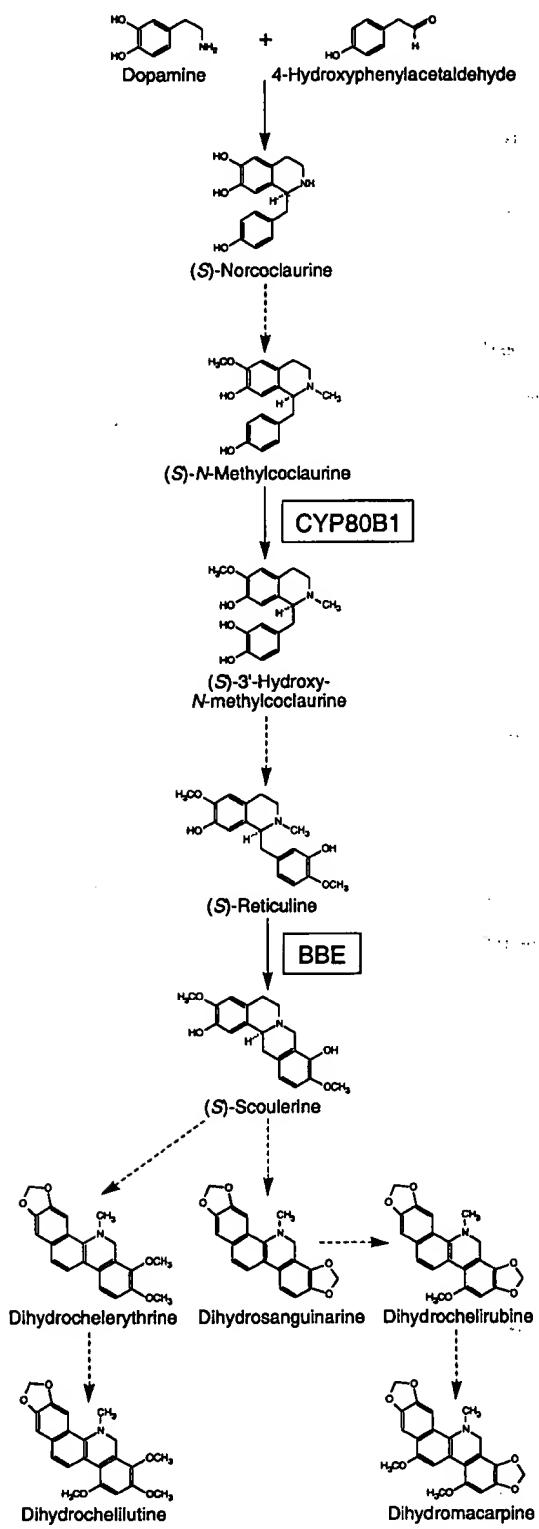


Figure 1. Biosynthesis of the major benzophenanthridine alkaloids found in California poppy (*Eschscholzia californica*) cell cultures showing the sites of action of the berberine bridge enzyme (BBE) and (S)-N-methylclaurine 3'-hydroxylase (CYP80B1).

N-methyltransferase, and two additional P450-dependent hydroxylases to dihydrosanguinarine, the first alkaloid with the benzophenanthridine nucleus (Facchini, 2001). Another P450-dependent monooxygenase and an *O*-methyltransferase are involved in the final steps leading to dihydromacarpine and related intermediates. Dihydrobenzophenanthridine oxidase converts these alkaloids to their corresponding oxidation products, such as sanguinarine and macarpine.

Plant metabolic engineering is a relatively new field of research with the potential to create new opportunities for the improvement of plant metabolic, cellular, and physiological processes. In recent years, a number of impressive strategies for the genetic modification of several important plant metabolic pathways have been reported. These include the reduction of indole glucosinolate levels in *Brassica napus* seeds (Chavadej et al., 1994), the elevation of the α -tocopherol (vitamin E) content of *Arabidopsis* seeds (Shintani and DellaPenna, 1998), and the introduction of the entire β -carotene (provitamin A) biosynthetic pathway into rice endosperm cells (Ye et al., 2000). The first application of metabolic engineering to a plant alkaloid pathway involved the transformation of *Atropa belladonna*, which normally accumulates hyoscyanine, with the gene encoding hyoscyanine 6 β -hydroxylase (H6H) from *Hyoscyamus muticus* (Yun et al., 1992). Plants expressing the H6H transgene accumulated high levels of scopolamine, the H6H reaction product, demonstrating that alkaloid metabolism can be altered in transgenic plants. However, the metabolic engineering of plant alkaloid pathways has generally been restricted by the limited availability of cloned biosynthetic genes and the inability to genetically transform many alkaloid-producing species. Recently, several new genes encoding enzymes involved in benzylisoquinoline alkaloid biosynthesis have been reported (Facchini, 2001) and protocols for the genetic transformation of the benzylisoquinoline alkaloid-producing species opium poppy (*Papaver somniferum*) and California poppy have been established (Belny et al., 1997; Park and Facchini, 2000a, 2000b, 2000c). These developments have created the opportunity to metabolically engineer benzylisoquinoline alkaloid pathways in plants.

In this paper, we report the suppression of benzophenanthridine alkaloid biosynthesis in cell cultures of California poppy transformed with antisense BBE and antisense CYP80B1 constructs. The consequences of restricting metabolic flux into benzophenanthridine alkaloid biosynthesis at these two key points in the pathway were evaluated by two-dimensional ^1H -NMR and *in vivo* ^{15}N -NMR spectroscopy. Our results show that changes in metabolic flux through benzylisoquinoline alkaloid biosynthesis can affect the regulation of amino acid pools. Our data also provide new insight into the development of meta-

bolic engineering strategies that target benzylisoquinoline alkaloid pathways.

RESULTS

Suppression of BBE or CYP80B1 mRNAs Reduces Benzophenanthridine Alkaloid Accumulation in California Poppy Cell Cultures

Several independent, paromomycin-resistant callus lines were obtained from excised California poppy cotyledons exposed to *Agrobacterium tumefaciens* strain GV3101 harboring the 35S::GUS, 35S::antiBBE, or 35S::antiCYP80B1 constructs. The callus cultures were maintained on growth media containing paromomycin and timentin for several months to promote the selection of transgenic cells and the complete elimination of *A. tumefaciens*. PCR performed using genomic DNA isolated from each paromomycin-resistant cell line and primers specific to sequences in the *NtPII* selectable marker gene consistently produced a single amplicon with the expected size of 823 bp. No amplicons were obtained using genomic DNA isolated from wild-type California poppy cell cultures. Suspension cultures were subsequently initiated from several cell lines and eventually transferred to growth media lacking antibiotics. This last step was necessary because benzophenanthridine alkaloid accumulation in California poppy cultures is inducible by antibiotics (Schumacher et al., 1987). All experiments were performed several months after the establishment of antibiotic-free cell suspension cultures.

Northern-blot hybridization analysis using radio-labeled sense RNA probes demonstrated the presence of antisense BBE and antisense CYP80B1 transcripts in California poppy cell cultures transformed with 35S::antiBBE and 35S::antiCYP80B1, respectively (Fig. 2). Alternatively, the use of antisense RNA probes showed a dramatic reduction in BBE and CYP80B1 mRNA levels in some transgenic cell lines expressing 35S::antiBBE and 35S::antiCYP80B1, respectively, compared with control cultures expressing 35S::GUS (Fig. 2; cell lines BBE-A1 and CYP80B1-A1). BBE mRNAs were nearly undetectable in some transgenic cell lines, such as BBE-A1, that produced high levels of antisense BBE transcripts. Although CYP80B1 mRNA levels were extensively reduced in some transgenic cell lines that produced high levels of antisense CYP80B1 transcripts, such as CYP80B1-A1, the reduction was never as substantial as that found in BBE-A1 (Fig. 2) and other antisense BBE cell lines. However, BBE and CYP80B1 mRNA levels were not reduced in all cell lines with abundant antisense BBE or antisense CYP80B1 transcripts, respectively (Fig. 2; cell lines BBE-A2 and CYP80B1-A2). As expected, BBE activity was similar in the BBE-A2 (16 ± 8 pkat mg⁻¹ protein) and control (19 ± 7 pkat mg⁻¹ protein) cell lines, but was below the

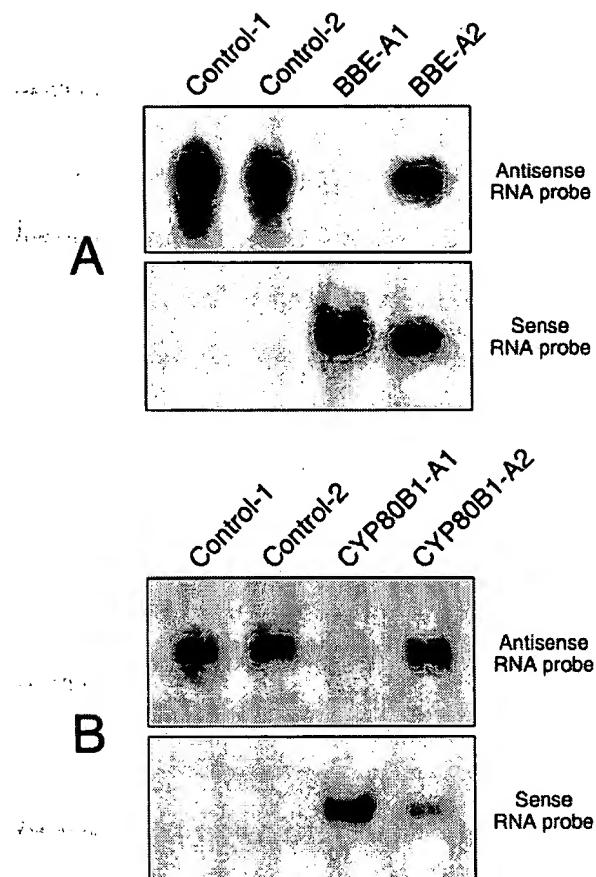


Figure 2. RNA gel blot analysis showing the accumulation of sense and antisense BBE and CYP80B1 transcripts in California poppy cell suspension cultures transformed with 35S::GUS (control-1 and control-2), 35S::antiBBE (BBE-A1 and BBE-A2), and 35S::anti-CYP80B1 (CYP80B1-A1 and CYP80B1-A2) constructs. Total RNA was extracted, and 15 μ g was fractionated on a 1% (w/v) formaldehyde agarose gel, transferred to a nylon membrane, and hybridized at high stringency with 32 P-labeled BBE or CYP80B1 sense or antisense RNA probes. Gels were stained with ethidium bromide before blotting to ensure equal loading.

detection limit of the assay in the BBE-A1 cell line (data not shown).

GUS transcripts were abundant in control cultures transformed with 35S::GUS. Moreover, BBE and CYP80B1 mRNA levels were identical to those in wild-type cultures (data not shown). CYP80B1 transcript levels in the BBE-A1 and BBE-A2 cell lines were also identical to those in wild-type cultures. Similarly, BBE mRNA levels in the CYP80B1-A1 and CYP80B1-A2 cell lines were the same as those in wild-type cultures. Transcript levels of another alkaloid biosynthetic gene, Tyr/dopa decarboxylase, were identical in control, BBE-A1, and CYP80B1-A1 transgenic cell lines (data not shown).

Cell lines transformed with 35S::GUS exhibited the same red color seen in wild-type California poppy cell cultures. However, transgenic cell lines that

showed a substantial reduction in BBE and CYP80B1 mRNA levels (i.e. BBE-A1 and CYP80B1-A1) did not possess the red pigmentation found in wild-type and control cultures. HPLC analysis showed that benzophenanthridine alkaloid levels were substantially reduced in cell lines that displayed high levels of antisense BBE or antisense CYP80B1 transcripts and low levels of BBE and CYP80B1 mRNAs, respectively, compared with controls (Fig. 3). No new peaks were

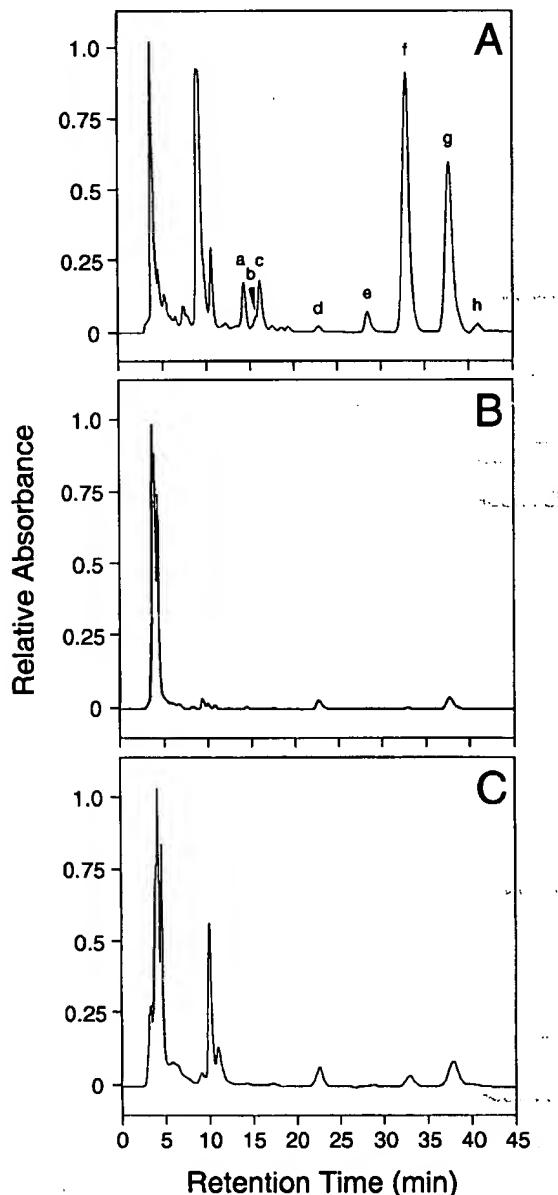


Figure 3. HPLC elution profiles of extracts from California poppy cell suspension cultures transformed with 35S::GUS (A, control-1), 35S::antiBBE (B, BBE-A1), and 35S::antiCYP80B1 (C, CYP80B1-A1) constructs. Peaks correspond to: a, chelilutine and chelirubine; b, chelerythrine and sanguinarine; c, marcarpine; d, dihydrochelilutine; e, dihydrochelerythrine; f, unknown alkaloid with $m/z = 338$; g, dihydromarcarpine; and h, dihydrosanguinarine.

apparent on HPLC chromatograms of extracts from cell lines with low levels of BBE or CYP80B1 mRNAs and significantly reduced total alkaloid accumulation compared with controls (Fig. 3). In particular, a peak corresponding to (S)-reticuline was not detected on HPLC chromatograms of alkaloid extracts from the BBE-A1 cell line. The extraction of (S)-reticuline using the protocol described herein was confirmed by adding authentic (S)-reticuline to some samples as an internal standard. Moreover, (S)-reticuline was not detected in the original methanol extracts (data not shown).

The total benzophenanthridine alkaloid content of the BBE-A1 cell line was 8- to 10-fold lower than that of control cells transformed with 35S::GUS (Table I). Similarly, the total alkaloid content of the CYP80B1-A1 cell line was 3- to 4-fold lower than that of controls (Table I). The level of most individual alkaloids was reduced in both the BBE-A1 and CYP80B1-A1 cell lines compared with controls, with the exception of dihydrochelilutine, which was present at marginally higher levels in cultures exhibiting a reduced abundance of BBE and CYP80B1 mRNAs (Table I). Cell lines that produced BBE and CYP80B1 antisense mRNAs but did not show a reduction in the corresponding sense mRNA levels (i.e. BBE-A2 and CYP80B1-A2) displayed total and individual levels of alkaloid accumulation that were not significantly different from those of controls (Table I).

Suppression of Benzophenanthridine Alkaloid Biosynthesis Reduces the Growth Rate of California Poppy Cell Cultures

Transgenic cell lines with low levels of BBE or CYP80B1 mRNAs, and significantly reduced total alkaloid accumulation, displayed growth rates that were considerably slower than those of control cultures (Fig. 4). The growth rates of control cultures transformed with 35S::GUS were not significantly different from those of wild-type cultures (data not shown). Similarly, the growth rates of the BBE-A1 and CYP80B1-A1 cell lines were not significantly different from each other (Fig. 4).

Antisense RNA-Mediated Reduction in BBE and CYP80B1 mRNA Levels Increases Free Amino Acid Pools in California Poppy Cells

Two-dimensional ^1H -NMR spectroscopy showed that the cellular pools of several amino acids were larger in cultures (i.e. BBE-A1 and CYP80B1-A1) exhibiting low levels of BBE or CYP80B1 mRNAs and significantly reduced total alkaloid accumulation (Fig. 5). Specifically, cellular pools of Ala, Leu, Phe, Thr, and Val were larger in cultures with reduced alkaloid levels compared with controls. In contrast, the relative abundance of Tyr appeared to be only

Table I. Accumulation of major benzophenanthridine alkaloids in cell suspension cultures of *E. californica* transformed with 35S::GUS (control-1 and control-2), 35S::antiBBE (BBE-A1 and BBE-A2), and 35S::antiCYP80B1 (CYP80B1-A1 and CYP80B1-A2)

Alkaloid	Cell Line					
	Control-1	Control-2	BBE-A1	BBE-A2	CYP80B1-A1	CYP80B1-A2
nmol g ⁻¹ (dry wt)						
Macarpine ^a	40.7 ± 47.1	14.5 ± 10.6	Trace	2.4 ± 2.7	7.2 ± 11.7	24.8 ± 16.5
Dihydromacarpine	278.9 ± 38.2	224.4 ± 128.5	55.3 ± 13.2	297.1 ± 175.7	76.3 ± 92.1	246.1 ± 141.4
Chelerythrine	3.7 ± 4.5	0.7 ± 0.7	Trace	2.4 ± 2.7	4.3 ± 4.0	0.7 ± 0.7
Dihydrochelerythrine	8.3 ± 0.7	8.0 ± 4.1	Trace	Trace	10.1 ± 5.5	10.0 ± 9.5
Chelilutine/chelirubine	28.4 ± 27.9	11.3 ± 8.3	2.8 ± 2.8	7.1 ± 7.8	3.7 ± 2.0	12.1 ± 9.8
Dihydrochelilutine	10.9 ± 4.1	7.0 ± 3.7	26.8 ± 6.8	109.7 ± 73.7	73.8 ± 75.8	193.7 ± 99.7
Sanguinarine	1.1 ± 1.5	0.4 ± 0.2	Trace	1.3 ± 0.7	2.1 ± 2.1	6.0 ± 9.9
Dihydrosanguinarine	10.9 ± 3.6	9.7 ± 7.4	Trace	Trace	4.4 ± 3.9	22.1 ± 38.3
m/z = 338 ^b	484.4 ± 132.4	413.7 ± 245.8	0.6 ± 0.2	113.8 ± 161.4	57.1 ± 36.4	127.7 ± 38.0
Total ^c	868.7 ± 248.6	690.0 ± 408.3	89.5 ± 21.1	544.5 ± 377.8	250.6 ± 207.6	627.2 ± 209.4

^a Values represent the mean ± SD of three independent experiments.

^b Unknown alkaloid with a M_r of 338.

^c Represents the sum of all major alkaloids.

marginally higher in the BBE-A1 and CYP80B1-A1 cell lines compared with controls (Fig. 5). Cellular levels of several carbohydrates including Suc, α-Glc, β-Glc, and malate were similar in the BBE-A1, CYP80B1-A1, and control lines. In vivo ¹⁵N-NMR spectroscopy also showed that the accumulation of detectable ¹⁵N-labeled amino acids, including α-amino acids, Ala, and Gln, was higher in cell lines exhibiting low levels of BBE or CYP80B1 mRNAs, and significantly reduced total alkaloid accumulation (Fig. 6). Two-dimensional ¹H-NMR and noninvasive ¹⁵N-NMR also showed a substantial increase in the accumulation of γ-aminobutyrate (GABA) in transgenic cell lines with a reduced accumulation of benzophenanthridine alkaloids compared with controls (Figs. 5 and 6). An increase in the cellular pool size of several amino acids in the BBE-A1 and CYP80B1-A1 cell lines, compared with control cultures, was con-

firmed by direct HPLC analysis (data not shown). Using this method, Tyr levels were found to be less than 2-fold higher in antisense-suppressed cell cultures compared with controls.

DISCUSSION

Constitutive expression of antisense BBE or anti-sense CYP80B1 genes in transgenic California poppy cell cultures yielded several lines with high levels of antisense transcripts. In approximately 50% of these lines, corresponding sense mRNA levels were substantially reduced compared with controls transformed with 35S::GUS. In the remaining cell lines, little change in sense mRNA levels was detected relative to controls. Two transgenic lines, one displaying nearly undetectable and the other relatively normal sense mRNA levels, were selected from each set of cell lines producing antisense transcripts. Two transgenic control lines were also selected at random. The BBE-A1 and CYP80B1-A1 cell lines accumulated low to undetectable levels of BBE and CYP80B1 mRNAs, whereas the BBE-A2 and CYP80B1-A2 cell lines displayed mRNA levels similar to those found in controls (Fig. 2). The suppression of BBE mRNA accumulation in the BBE-A1 cell line was also accompanied by a substantial reduction in BBE activity. CYP80B1 activity was not measured because an enzymatic substrate was not available. However, it can be assumed that CYP80B1 activity was also substantially reduced in transgenic cell lines with low levels of CYP80B1 mRNA compared with controls.

The suppression of BBE and CYP80B1 mRNAs in transgenic California poppy cell cultures resulted in a dramatic reduction in the accumulation of benzophenanthridine alkaloids (Fig. 3; Table I). BBE activity was below the detection limit of the assay. However, the accumulation of alkaloids at one-tenth the control level suggests that BBE activity was reduced by about 90% compared with controls. Alkaloid ac-

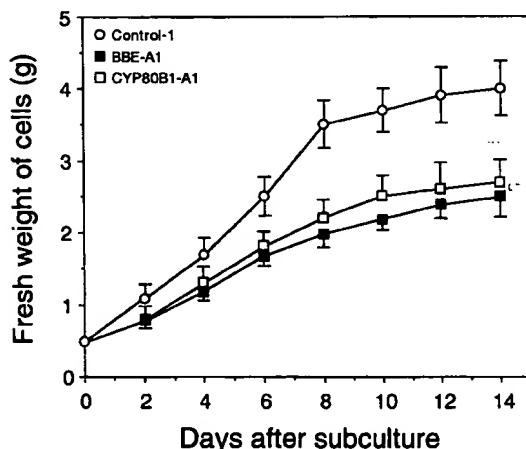


Figure 4. Growth rate of California poppy cell suspension cultures transformed with 35S::GUS (control-1), 35S::antiBBE (BBE-A1), and 35S::antiCYP80B1 (CYP80B1-A1) constructs. Each value represents the mean ± SD of the fresh weight of cells per flask from three independent experiments.

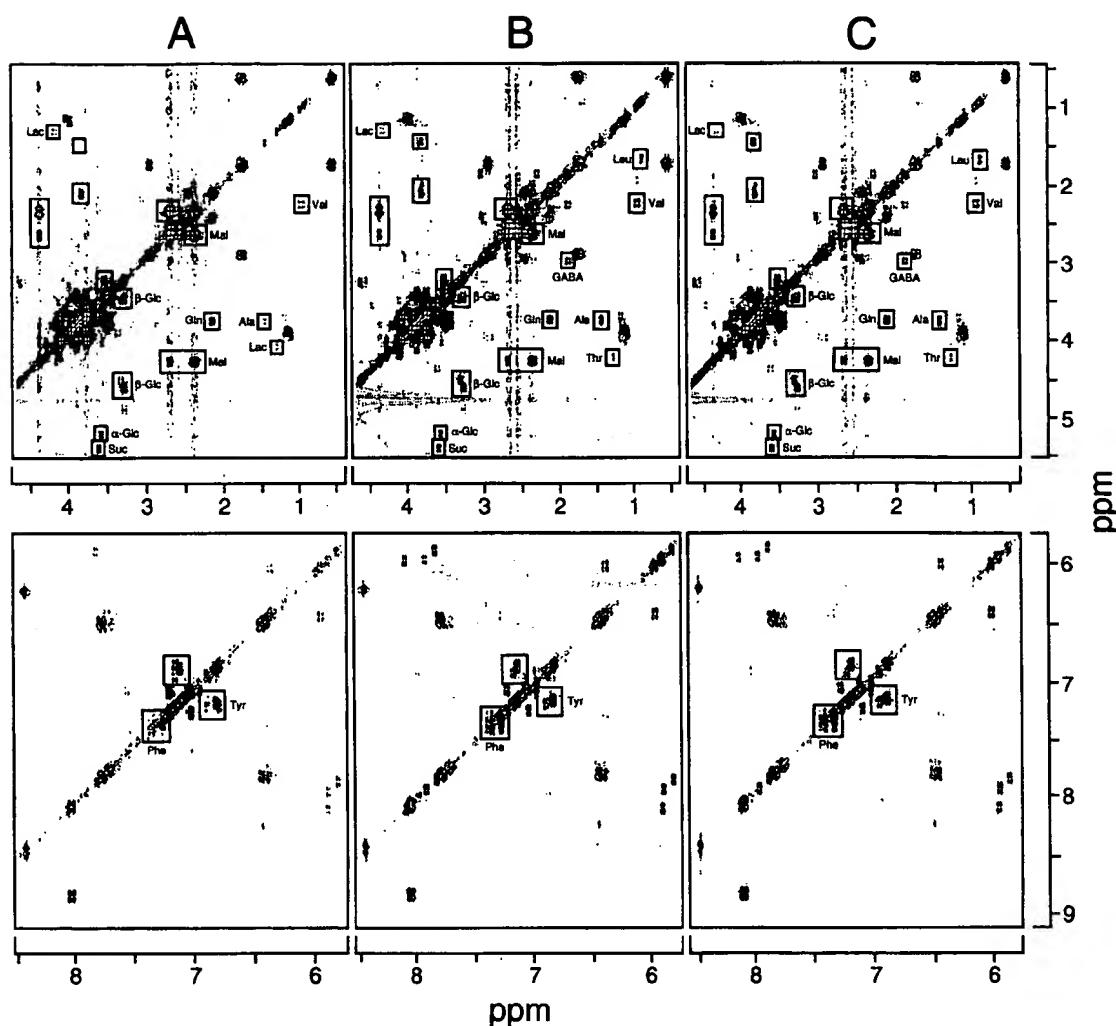


Figure 5. Two-dimensional ^1H -NMR spectra of ethanol extracts from California poppy cell suspension cultures transformed with 35S::GUS (A, control-1), 35S::antiBBE (B, BBE-A1), and 35S::antiCYP80B1 (C, CYP80B1-A1) constructs. The spectra were normalized using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. Lac, Lactate; Mal, malate.

cumulation in transgenic lines that displayed control levels of BBE or CYP80B1 mRNAs was essentially the same as that in control cultures. Transgenic cell lines expressing 35S::GUS were used as controls to account for any metabolic or cellular alterations caused by the transformation process. Previously, we showed that the transformation of California poppy and opium poppy plants and root cultures did not alter the growth rate, anatomy, or benzylisoquinoline alkaloid content of these systems (Park and Facchini, 2000a, 2000b, 2000c). Moreover, transcript levels for other alkaloid biosynthetic genes were virtually identical in control and antisense-suppressed cell lines. These data indicate that the reduced accumulation of benzophenanthridine alkaloids in the BBE-A1 and CYP80B1 cell lines was specifically due to the silencing, or partial-silencing, of BBE or CYP80B1 mRNAs.

Several points about the effect of the antisense RNA-mediated suppression of benzophenanthridine

alkaloid biosynthesis should be noted. First, a decrease in either BBE or CYP80B1 mRNA levels produced a similar reduction in alkaloid accumulation. Second, the metabolic intermediates (*S*)-reticuline and (*S*)-*N*-methylcoclaurine, which serve as substrates for BBE and CYP80B1, respectively, did not accumulate in antisense-suppressed cells (Fig. 3). The antisense suppression of BBE and CYP80B1 mRNAs caused an overall reduction in benzophenanthridine alkaloid accumulation, but the absence of additional peaks on HPLC chromatograms shows that pathway intermediates did not accumulate. Third, the silencing of BBE or CYP80B1 mRNAs resulted in a significant reduction in the growth rate of the cell cultures. The reduced growth rate occurred in all antisense-suppressed cell lines (Fig. 4), but not in transgenic cell lines exhibiting control levels of BBE and CYP80B1 mRNAs (data not shown). The reduced

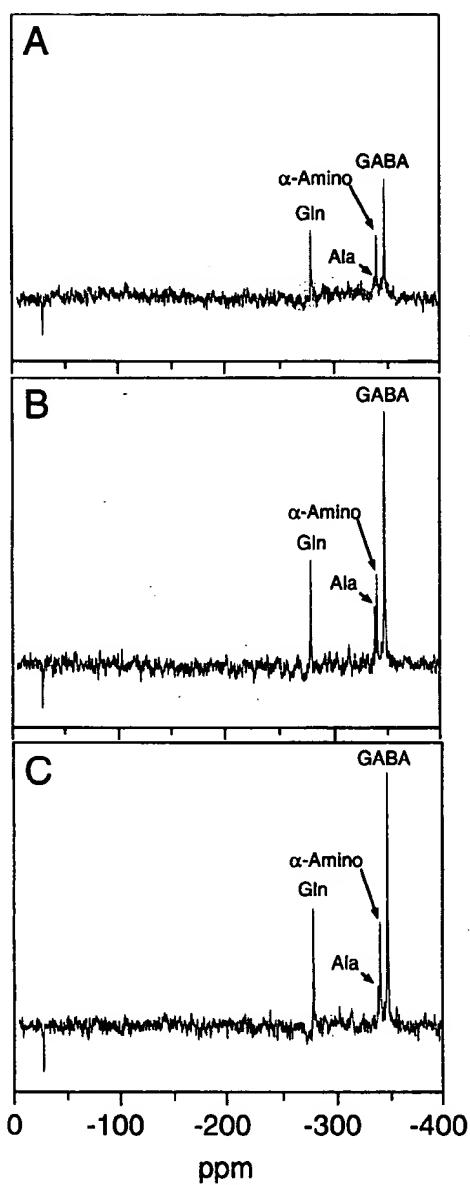


Figure 6. In vivo ^{15}N -nuclear magnetic resonance spectra of California poppy cell suspension cultures transformed with 35S::CUS (A, control-1), 35S::antiBBE (B, BBE-A1), and 35S::antiCYP80B1 (C, CYP80B1-A1) constructs. Cultures were grown in standard B5 media containing K^{15}NO_3 rather than the corresponding ^{14}N -containing compound.

growth rate might have resulted from the low-level accumulation of cytotoxic pathway intermediates.

We suggest three hypotheses for the lack of a substantial accumulation of pathway intermediates in antisense-suppressed cell lines. The first involves feedback inhibition by one or more alkaloid intermediates on early biosynthetic enzymes. However, the inhibition of enzymes involved in benzylisoquinoline alkaloid biosynthesis by pathway intermediates or end products has not been reported. The second con-

cerns the putative degradation of alkaloid intermediates by an as-yet-uncharacterized mechanism. A third hypothesis raises the possibility that benzylisoquinoline alkaloid biosynthetic enzymes operate as part of a metabolon, or metabolic channel. Although direct interactions between alkaloid biosynthetic enzymes have not yet been demonstrated, the existence of enzyme complexes has been detected in flavonoid biosynthesis (Burbulis and Winkel-Shirley, 1999; He and Dixon, 2000). In particular, He and Dixon (2000) have suggested that the association of an O-methyltransferase involved in isoflavonoid biosynthesis might result in an altered regiospecificity, compared with that exhibited by the isolated enzyme, by facilitating the direct presentation of substrates into the active site. Similarly, removal of components, such as BBE or CYP80B1, from a putative metabolic complex might preclude the accumulation of pathway intermediates because of the lack of coordination between integrated active sites of sequential biosynthetic enzymes. In addition, the apparent spatial segregation of benzophenanthridine alkaloid biosynthesis and end-product sequestration within a cell might also contribute to the lack of (*S*)- N -methylcoclaurine or (*S*)-reticuline accumulation. Specifically, benzylisoquinoline alkaloid end products, such as sanguinarine, accumulate in the vacuole (Kutchan et al., 1986), whereas BBE has been shown to contain a signal peptide (i.e. targeting the endoplasmic reticulum) and a vacuolar sorting determinant (Bird and Facchini, 2001). Thus, an engineered metabolic block in benzylisoquinoline alkaloid biosynthesis might sufficiently disrupt the normal metabolic and intracellular transport architecture to prevent the accumulation of pathway intermediates or end products. The inherent cytotoxicity of alkaloids is at least partially responsible for the extensive subcellular compartmentation of many biosynthetic pathways to sequester intermediates and end products away from the cytosol (De Luca and St-Pierre, 2000). The reduced growth rate of antisense-suppressed California poppy cells might be caused by the impaired metabolic flux of (*S*)- N -methylcoclaurine and (*S*)-reticuline, leading to the accumulation of these alkaloids to marginally cytotoxic levels in an improper subcellular environment. Clearly, a better understanding of the subcellular localization of biosynthetic enzymes, the trafficking of intermediates, and the mechanisms that control flux, is essential to design rational metabolic engineering strategies that target benzylisoquinoline alkaloid pathways.

The expanding collection of biosynthetic genes should contribute to an improved understanding of the metabolic mechanisms that regulate benzylisoquinoline alkaloid biosynthesis. For example, constitutive expression of the scoulerine 9-O-methyltransferase (SMT) gene in *Coptis japonica* cell cultures resulted in 20% higher SMT activity and a small

increase in the accumulation of protoberberine alkaloids (Sato et al., 2001). SMT diverts (*S*)-scoulerine toward protoberberine, rather than benzophenanthridine, alkaloid biosynthesis. Expression of SMT in cultured California poppy cells led to a diversion of metabolic flux toward the protoberberine alkaloid columbamine and away from benzophenanthridine alkaloids. Columbamine is a novel product in this system since California poppy cell cultures do not possess native SMT activity. Hence, the modulation of a branch-point enzyme in benzylisoquinoline alkaloid biosynthesis can alter the flux of pathway intermediates toward native or novel products.

Relatively little attention has been focused on the relationship between benzylisoquinoline alkaloid and primary metabolic pathways. Certainly, an important consideration for any metabolic engineering strategy is the availability of primary metabolic precursors. The role of Tyr as the precursor to the benzylisoquinoline alkaloid pathway suggests that the regulation of the shikimate and aromatic amino acid pathways might contribute significantly to the ability of California poppy cell cultures to produce substantial quantities of products, such as sanguinarine and macarpine. NMR spectroscopy is a technique that can provide valuable insights into the integration and regulation of plant metabolism through both *in vitro* and *in vivo* measurements. We used two-dimensional ¹H-NMR and noninvasive ¹⁵N-NMR to obtain a broad profile of metabolic alterations caused by antisense mRNA-mediated suppression of benzophenanthridine alkaloid biosynthesis. Recent applications of ¹H-NMR include an analysis of the ligands of barley root exudates (Fan et al., 1997) and the metabolic composition of tomato fruits (Noteborn et al., 2000). Two-dimensional ¹H-NMR of ethanol extracts from control and antisense-suppressed California poppy cell cultures showed the relative abundance of several amino acids, organic acids, and sugars. Similar internal concentrations of Suc, Glc, and malate suggest that many basic metabolic processes were not affected by the antisense suppression of BBE or CYP80B1 (Fig. 5). In contrast, cellular pools of several amino acids, especially Ala, Leu, Phe, Thr, Val, and to a lesser extent Gln were larger in antisense-suppressed cells compared with controls. An increase in the abundance of amino acids in antisense-suppressed cell lines might be expected because of the reduced flux of nitrogen into benzophenanthridine alkaloids. The increased abundance of Ala, Gln, and α -amino acids was confirmed by noninvasive ¹⁵N-NMR (Fig. 6). *In vivo* ¹⁵N-NMR has frequently been used to detect amino acids (Robinson et al., 1991) and some secondary metabolites, such as nicotine, agropine, and conjugated polyamines (Ford et al., 1994). Tyr levels also increased in antisense-suppressed cell lines compared with controls, but the increase was less than 2-fold. However, the elevated Tyr pool cannot account for the decrease in the ac-

cumulation of Tyr-derived benzylisoquinoline alkaloids in cell lines BBE-A1 and CYP80B1-A1. A decrease in the flux of Tyr into benzophenanthridine alkaloid biosynthesis clearly alters the steady-state levels of several amino acids. The increase in GABA levels in antisense-suppressed cultures should also be noted (Figs. 5 and 6). GABA is present in all plant tissues and accumulates under stress conditions, and has been shown to function as a major nitrogen sink in non-stressed carrot cell cultures (Robinson et al., 1991) and in the short-term maintenance of pH homeostasis (Carroll et al., 1994).

We have shown that the transformation of California poppy cell cultures with antisense BBE or antisense CYP80B1 constructs can suppress the accumulation of benzophenanthridine alkaloids, which in turn affects cellular growth rate and amino acid metabolism. Our data provide insight into the complex regulation of benzylisoquinoline alkaloid biosynthesis and the integral relationship between amino acid and alkaloid metabolism. Much remains to be learned about the control of benzylisoquinoline alkaloid pathways before we can routinely design rational metabolic engineering strategies. A proposed model suggesting that a plant cell can be engineered to accumulate valuable benzylisoquinoline alkaloid intermediates using an antisense mRNA-mediated approach (Kutchan, 1995) does not appear to be feasible in some cases because of the complex and poorly understood overall metabolic regulation of the pathway.

MATERIALS AND METHODS

PCR

Plant genomic DNA was extracted as described by Edwards et al. (1991). Tissues (50 mg fresh weight) were homogenized in 200 μ L of extraction buffer (0.5% [w/v] SDS, 250 mM NaCl, 100 mM Tris-HCl, pH 8.0, and 25 mM EDTA) and centrifuged. The supernatant was mixed with an equal volume of isopropanol, incubated on ice for 5 min, and then centrifuged. The pellet was dried at 60°C for 10 min, and resuspended in 100 μ L of TE buffer (10 mM Tris-HCl, pH 7.4, and 1.0 mM EDTA). PCR was performed for 30 thermal cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min). BBE and CYP80B1 were amplified from California poppy (*Eschscholzia californica* Cham.) genomic DNA using primers corresponding to the beginning and end of each open reading frame (BBE, 5'-ATGGAAACAAAAACTCCC-3' and 5'-CTATATTACAACCTCTCC-3' [Dittrich and Kutchan, 1991]; CYP80B1, 5'-ATGGAGGT-TGTCACAGTA-3' and 5'-TCAAACCCCTTGATTAGG-3' [Pauli and Kutchan, 1998]). PCR was also used to test the transformation of cell cultures using primers specific to sequences in *NPTII* (5'-CAAGATGGATTGCACGCA-3' and 5'-TCACCCGAAGAACCGTC-3').

Construction of Transformation Vectors

BBE and *CYP80B1* coding regions were re-amplified by PCR using primers designed to add *KpnI* and *Sall* restriction sites to the 5' and 3' ends, respectively, of each clone. The PCR products were inserted into the pBI102 binary vector (Facchini et al., 1996) between *KpnI* and *Sall* sites to allow expression of antisense RNA driven by the cauliflower mosaic virus 35S promoter. Each construct was mobilized in *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmid pMP90 (Koncz and Schell, 1986). *A. tumefaciens* cultures were grown at 28°C on a gyratory shaker at 180 rpm in liquid Luria-Bertani medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl, pH 7.0), containing 50 mg L⁻¹ kanamycin, to mid-log phase ($A_{600} = 0.5$). The bacterial cells were collected by centrifugation for 10 min at 270g, and resuspended at a cell density of $A_{600} = 1.0$ in liquid inoculation medium (B5 salts and vitamins and 20 g L⁻¹ Suc).

Production of Transgenic Cell Cultures

Seeds of California poppy (Richters Herbs, Goodwood, Canada) were surface-sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite solution for 10 min, rinsed three times in sterilized water, and germinated on basal media consisting of B5 salts and vitamins (Gamborg et al., 1968), pH 5.8, solidified with 0.8% (w/v) Phytagar (Gibco, Burlington, Canada). All plant tissues were maintained in a growth chamber at 25°C under standard cool-white fluorescent tubes (Sylvania Gros-Lux Wide Spectrum, Mississauga, Canada) with a flux rate of 35 $\mu\text{mol s}^{-1} \text{m}^{-2}$ and a 16-h photoperiod, unless otherwise noted. Excised cotyledons from 7-d-old seedlings were isolated by longitudinal bisection of the hypocotyl. Cotyledons were immersed for 15 min in the *A. tumefaciens* suspension cultures containing the various transformation constructs, blotted dry on sterile filter paper, and incubated in the dark at 25°C on callus induction media (B5 salts and vitamins, 30 g L⁻¹ Suc, 1.0 mg L⁻¹ 2,4-dichlorophenoxyacetic acid, and 8 g L⁻¹ Phytagar). After 2 d of cocultivation with *A. tumefaciens*, the cotyledons were transferred to callus induction media containing 50 mg L⁻¹ paromomycin and 200 mg L⁻¹ timentin. After 12 weeks, the callus was transferred to liquid B5 media containing 1.0 mg L⁻¹ 2,4-dichlorophenoxyacetic acid, 50 mg L⁻¹ paromomycin, 200 mg L⁻¹ timentin, and 1 g L⁻¹ casein hydrolysate to produce suspension cultures. Cell suspensions were maintained in 30 mL of media in 125-mL Erlenmeyer flasks on a gyratory shaker at 120 rpm. The growth of suspension cultures was measured as the total fresh weight of tissue in one 125-mL Erlenmeyer flask.

RNA Gel-Blot Hybridization

Total RNA for gel-blot hybridization analysis was isolated using the method of Logemann et al. (1987). Fifteen micrograms was fractionated on a 1.0% (v/v) formaldehyde agarose gel before transfer to a nylon membrane. Duplicate blots were hybridized with sense or antisense

RNA probes transcribed from linearized California poppy *BBE* and *CYP80B1* open reading frames in pBluescript using T3 and T7 RNA polymerases, [α -³²P]UTP, and an in vitro transcription kit (Stratagene, La Jolla, CA). Hybridization was performed at 70°C in 0.25 mM sodium phosphate-buffer, pH 8.0, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA. The blot was washed at 70°C, twice with 2× SSC and 0.1% (w/v) SDS and twice with 0.2× SSC and 0.1% (w/v) SDS (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), and autoradiographed with an intensifying screen at -80°C for 24 h.

HPLC Analysis

California poppy cell cultures were frozen in liquid N₂, ground to a fine powder using a mortar and pestle, and extracted with methanol in a boiling water bath for 15 min. Extracts were reduced to dryness under vacuum, dissolved in 1.0 M sodium carbonate/bicarbonate (3:2, w/w), pH 10.0, and extracted three times with ethyl acetate. Pooled ethyl acetate fractions were reduced to dryness and the residue taken up in 1 mL of methanol. Extracts were analyzed using a System Gold 126 HPLC and 128 photodiode array detector (Beckman-Coulter, Mississauga, Canada). Alkaloids were separated at a flow rate of 0.75 mL min⁻¹ on a C₁₈ reverse phase column (4.6 × 250 mm, Ultrasphere, Beckman-Coulter) using methanol:water (3:1, v/v) containing 0.1% (v/v) triethylamine. The identity of peaks corresponding to various benzophenanthridine alkaloids was initially determined by liquid chromatography-mass spectroscopy. Subsequently, peaks were routinely analyzed by comparison of UV spectra and retention times with those of identified alkaloids. The expected retention time of (S)-reticuline was determined using an authentic standard.

Enzyme Assay

Two grams of cultured cells was ground to a fine powder under liquid N₂ using a mortar and pestle and extracted in 50 mM Gly-NaOH, pH 8.9. Polyvinylpolypyrrolidone was added to remove phenolic compounds and the extract was desalted on a PD-10 column (Amersham-Pharmacia, Uppsala). Reaction mixtures consisting of 1 mM (S)-reticuline and 500 μL of the enzyme extract were incubated at 30°C for 1 to 2 h. Reactions were stopped by the addition of 10 μL of 1.0 N NaOH, and 100 μL was fractionated on a C₁₈ reverse phase column (4.6 × 250 mm, Ultrasphere, Beckman-Coulter) using methanol:water (1:1, v/v) containing 0.1% (v/v) triethylamine as the mobile phase. The (S)-scoulerine peak was identified by comparison of its UV spectrum and retention time with those of an authentic standard.

¹⁵N- and Two-Dimensional ¹H-NMR Spectroscopy

Whole-cell extracts for two-dimensional ¹H-NMR analysis were prepared by grinding 100 mg of lyophilized California poppy cell cultures in three 10-mL aliquots of 80% (v/v) ethanol. After centrifugation of the pooled ex-

tracts, the supernatant was reduced to 5 mL under vacuum, de-ionized using 1 mL of Chelex-100, and lyophilized. The sample was dissolved in 100% (v/v) D₂O to reduce the resonance for water. Nitrogen-15 NMR spectra were determined using cell cultures grown for 3 d in media containing 99.9% (w/w) K¹⁵NO₃ rather than the equivalent ¹⁴N-containing compound. NMR(NMR) studies were performed with a Bruker AM400 widebore NMR spectrometer equipped with a 10-mm multinuclear broadband probe. NMR spectra were recorded at the following frequencies: ¹H (400 MHz) and ¹⁵N (40 MHz). For in vivo measurements, cells were perfused in the NMR magnet by recirculating oxygen-saturated media.

Amino Acid Analysis

Cultured cells (1 g) were lyophilized and ground in 100% methanol (10:1 [v/w]). The homogenate was incubated at 60°C for 30 min and then centrifuged for 15 min at 12,000g. The supernatant was collected, and the pellet was extracted once more with 50% (v/v) methanol. The combined extracts were reduced to dryness and redissolved in 75 μL of dilution buffer containing 100 mM NaHCO₃ and 100 mM H₃BO₃, pH 8.5. Twenty microliters of the resuspended solution was mixed with 20 μL of 9-fluorenylmethyl chloroformate (20.7 mg mL⁻¹) and incubated at room temperature for 10 min to generate fluorescent amino acid derivatives. After extraction of the free fluorescent dye in 60 μL of pentane:ethyl acetate (8:2, v/v), 20 μL of the aqueous phase was subjected to HPLC. Amino acids were separated on an AminoTag column (Varian, Sugarland, TX) at a flow rate of 1.4 mL min⁻¹ using the following solvent system: A, 50 mM sodium acetate buffer, pH 4.2, 20% (v/v) acetonitrile; B, 50 mM sodium acetate buffer, pH 4.2, 70% (v/v) acetonitrile (100% A ramped to 25% B over 22 min, 75% B over 20 min, 100% B over 1 min, and 100% A over 2 min). Amino acid derivatives were quantified using a Prostar 363 fluorescence detector (Varian) with excitation at 264 nm and emission at 340 nm.

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